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13. ABSTRACT ***(Maximum 200 Words)***

Our aim is to identify epitopes on the surface of prostate cells that are largely or exclusively tissue- or tumor-specific. This is done by expansion of T cells reactive for HLA-restricted, established tumor lines from the peripheral blood cells and tumor-infiltrating lymphocytes of cancer patients. Towards this end, we established a reagent bank of patient material that includes tissue, cells, and serum. We successfully and stably gene-modified the established prostate cell line, LNCaP, so that it expresses HLA-A2 and HLA-A1. We successfully transfected two other cell lines with these genes, transiently but sufficiently, for our in vitro stimulation technique. Several potentially HLA-restricted, tumor-specific T cells have been identified and are currently being cultured by methods developed in our laboratory. In addition, we are performing an antibody-based method of antigen discovery that utilizes patient serum. Using this approach, several genes have been identified, some of which represent previously unreported, novel genes. Studies are underway to discern the exact epitope being recognized. Studies are also underway to assess the level of gene expression in normal vs tumor tissue from representative regions of the body.

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INTRODUCTION

Tumor-specific T cells, a crucial component of the immune system, have been demonstrated as able to recognize and respond to specific tumor antigens on allogeneic tumor cells in a MHC-restricted manner. Therefore, there is compelling evidence that a specific immune response in human is present against several different forms of cancer. Peripheral blood offers important benefits as a source for the generation of antigen-restricted CTL since it is easily collected from practically all cancer patients. There has been some evidence of using tumor-infiltrating lymphocytes co-cultured with irradiated tumor cells for the production of tumor-specific CTL, but there is a paucity of experimental data using this approach with peripheral blood and prostate tumor cell lines. We leukopheresed over twenty patients with prostate cancer and have on site several established prostate tumor cell lines. These lines were gene-modified to stably express two of the most common human leukocyte antigen (HLA) alleles occurring in North American men: HLA-A1 and –A2. Therefore, we had a source of patient material – from cells – to conduct mixed lymphocyte-tumor cell cultures for the generation of tumor-specific CTL. Also, we had a source of patient material – from sera – to use SEREX: serological identification of antigens by recombinant expression cloning. SEREX is an antibody-based approach to antigen discovery that proved much more valuable and successful than the T cell-based expression cloning. Expression cloning and SEREX were used to identify the distinct epitope on the tumor cell surface being recognized by the T-cell receptor on the CTL. Following screening of cDNA libraries by both cell- and serum-based approaches, specific genetic targets of recognition were identified. Such loci were sequenced to determine if a novel or previously identified epitope was detected. We did not have time to complete the final parts of the study: using the putative protein sequence, synthesizing peptides conforming to the HLA consensus

binding motif for that haplotype. We had intended that peptides would be screening by pulsing them exogenously onto HLA-matched T2 or autologous ebv-b cells and assessing if they stimulated antigen-specific CTL. Following this course, it was hoped that the epitope(s) recognized by the CTL or antibodies from patient sera could eventually be identified.

BODY

Task 1. To generate $CD8^+$ T cells specifically reactive at least for the stimulating prostate cell line in a MHC-restricted fashion.

a. Gene-modify prostate tumor cell lines for stable, high expression of HLA. (see below)
b. Perform assays to determine which mixed lymphocyte tumor cell (MLTC) conditions are preferred for the generation of antigen-specific, HLA-restricted cytotoxic T lymphocytes. (see below)
c. Produce and expand sufficient amounts of effector cells for expression cloning. This series of experiments has only begun recently.

We attempted to stably transfect the gene encoding HLA-A1 and HLA-A2 into the established cell lines, LNCaP, DU145 and PC3. Tables and Figures summarizing this work was included in the 1999 Annual Report. To reiterate, LNCaP was easy to stably transfect; that is, high levels of HLA-A2 expression were maintain following 10 passages or greater of the original transfectant line. DU145 and PC3 were difficult to stably transfect, as the level of expression and the precentage of cells positive for transgene expression decreased after a few passages (data not shown). It was not feasible to produce long-lasting HLA-A2-transfected DU145 and PC3 cells, and so we were unable to cryopreserve a large number of aliquots the highly expressing

initial passages. Our other alterative, transfecting the tumor lines a few days prior to their use for in vitro stimulations, proved unfeasible as well.

Because we have established LNCaP cells stably transfected with the HLA-A2 gene (LNCaP/A2), most of our MLTC experiments will involve this line. Should HLA-A2-modified DU145 and PC3 cells be needed, we will perform a transient transfection that should produce a high level of gene expression during MLTC coculturing as well as in ELISA.

Since setting up numerous 96-well culture conditions are both labor- and reagent-intensive, it was necessary to determine which MLTC conditions were more likely to produce HLA-restricted cloids. Therefore, for several experiments, 96-well plates were set up at various concentrations and ratios of cells. Representative assays were shown in the 1999 Annual Report. In summary, experiment one used LNCaP/A2 cells. Experiment two used PC3/A2 cells. Two 96-wells at each of the nine indicated ratios of PBMC and tumor cells were co-cultured for 10 days. Following this, microcultures were screened in singlicate for IFN-gamma secretion. Sixty microliters of each 200ul microculture was added to 5×10^4 HLA-A2 transfected prostate tumor cells. An equal volume was added to 5×10^4 of the parental tumor line. In general, for both experiments, plated fewer total cells resulted in specific cytokine secretion (defined as cytokine secretion against the transfected tumor line two times that of the parental line, and $\geq$100 pg/ml). The most successful ratio was determined as 10:1; either 1) 2×10^5 PBMC and 2×10^4 tumor cells, or 2) 2×10^5 PBMC and 4×10^4 tumor cells. We had hoped that optimizing MLTC conditions would permit us to set up experiments with a greater chance of generating specifically reactive cloids from the same initial number of established microwells. However, the inability to generate stable cloids generating sufficient levels of gene expression (in order to stimulate in

vitro allogeneic PBMC) made this method impractical, and accelerated our decision to continue the overall effort with an antibody-based approach.

Task 2. To identify the peptide epitope responsible for immune recognition by antigen-specific, HLA-restricted T lymphocytes.

a. Produce cDNA libraries of those prostate tumor cell lines for which CTL have been generated.
b. Use ELISPOT and ELISA to screen cDNA library for that fragment which confers reactivity to antigen-specific, HLA-restricted cytotoxic T lymphocytes.
c. Screen candidate peptides from putative protein sequence using CTL admixed with exogenous pulsed readout cells to determine peptide conferring reactivity.

As part of the effort to identify the epitope recognized by T cells stimulated in vitro with DU145/A2 cells), we generated a cDNA library from DU145 poly (A+) RNA. This was initially performed in lambda phage, a portion of which was converted to plasmid (via a helper phage). We found that 88% of the library's plasmids contained cDNA inserts and the size of the inserts ranged from 0.5 to 1.6 Kb. Thus, the quality of the cDNA library was good. To determine if the DU145 cDNA library contained prostate-specific genes, we performed PCR analysis to detect PSA and PSMA, as well as two housekeeping genes GAPDH and β-actin. Indeed, we demonstrated the presence of all four genes in the DU145 cDNA library.

As a prerequisite for screening the DU145 cDNA library for cDNAs which encode antigen(s) that are recognized by DU145/A2-specific T cells, we introduced one-half of the plasmid form this library into 293 cells. The 293 cells were then selected for stable transfectants, and 25 subfractionated $G418^R$ lines isolated. We grew up these stably-transfected 293 cells and

tested them for recognition by the DU145/A2-specific T cells. It was previously shown that the DU145/A2-specific T cells recognized DU145-derived epitopes in the context of both HLA-A1 and HLA-A2. Therefore, we transiently transfected 18 subfractionated lines with plasmid encoding HLA-A1, HLA-A2, or no insert. After 3 days, we co-cultivated these transfected sublines with two cloids of DU145/A2-specific T cells, collected supernatants after 48 hrs, and measured the IFN-γ released.

Using the aforementioned MLTC conditions for an initial screening, two cloids (#10 and #19) were identified as secreting IFN-gamma when co-cultured in an ELISA with DU145/A2 cells but not the DU145 parental line. We performed three independent screening assays on the 24 DU-series sublines (293 cells stably-transfected with DU145 cDNA). After transfecting with plasmid encoding HLA-A2, -A1, or vector alone, we co-cultivated these cells with an equal number of DU145/A2-specific effector cells (cloids 10 and 19) and assayed for IFNγ secretion by ELISA. Unfortunately, these assays were difficult to interpret because of the aberrant specificity of cloids 10 and 19 exhibited when co-cultivated with DU145 cells transfecting with plasmid encoding HLA-A2, -A1, or vector alone. For cloid 10, co-cultivation with DU145/A2 cells (positive control) resulted in IFN-gamma production in only one of the three assays. Cloid 19 effectors consistently released IFN-gamma when co-cultivated with untransfected DU145 cells, as well as DU145/A1 or -A2 transfectants. Further work is determined that this reactivity was non-specific. Due to the increasing use of SEREX, and the accompanying de-emphasis of the T cell approach, we did not generate other cloids stimulated in vitro with DU145/A2 and other transfected cell lines.

Using the methodology outlined in Figure 1 (see description below), we successfully generated a patient-derived reagent bank, consisting of tissue (Table 1a) and tumor infiltrating

lymphocytes (TILs) from prostate cancer patients (Table 1b). Most of the TIL were derived from biopsies, although a few were harvested from prostatectomy tissue. TIL were separated from tissue fragments and other contaminants via density centrifugation separation, following by cultivation in high-dose interleukin-2 (3000IU/ml). Four separate TIL lines were generated, although none at sufficient quantities to use with a T cell-based antigen discovery approach.

None of the cell lines or cloids that were generated (and described in the 1999 Annual Report) proved to have sufficient antigen-specific, HLA-A2-restricted lytic activity to warrant further analysis.

We accumulated a bank of patient serum during the study, derived from the whole blood that often accompanies tissue samples. As a result, we decided to supplement the T cell-based method of antigen discovery using a serological identification of antigens by SEREX technology. This approach utilized autologous patient sera to search for tumor antigens expressed in bacteria *Escherichia coli*, which are infected with lambda phages containing cDNA libraries prepared from fresh tumor tissues. Thus, this new approach bypassed both requirements for the pre-establishments of stable CTL clones or tumor-infiltrating lymphocytes (TIL), and established tumor cell lines. The use of SEREX technology in identification of human tumor antigens was been successful in many tumor types, including melanoma (1,2), renal cancer (1,3), astrocytoma (1), Hodgkin's disease (1,4), esophageal cancer (1), lung cancer (5,6), and colon cancer(7), and a number of tumor specific or associated antigens have been identified from these cancers. The tumor antigens identified by SEREX are immunogenic and are good candidates for cancer vaccines when the cognate T cell epitopes are discerned. The ability of the SEREX-identified antigen to be recognized by CTL was demonstrated in a recent study showing that the

peptide epitopes derived from a SEREX-identified tumor antigen NY-ESO-1 were recognized by CTL from a patient with high NY-ESO-1 antibody titers (8).

The overall SEREX approach is shown in Figure 2. Briefly, total cellular RNA was isolated from the cultured cells using a RNA isolation reagent. Complementary DNA expression libraries were constructed from two prostate cancer cell lines, LNCaP and PC3. Poly A+ mRNA was purified from the total cellular RNA isolated from these cells. The cDNA libraries were screened with a pool of sera from 10 patients with prostate cancer diagnosed at clinical stage D2. Membranes containing the recombinant phages were incubated with pooled patient sera, then incubated with an alkaline phosphatase (AP)-conjugated, Fc fragment-specific goat anti-human IgG. The positive plaques were visualized by staining the membranes with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate. Following 2-3 more rounds of subcloning, positive plaques were cloned to homogeneity.

The inital screening resulted in a detection of a total of 6 positive clones from LNCaP library and 11 positive clones from PC3 library. Subsequent analyses by restriction digestion, sequencing, and individual serum reaction revealed that the 6 LNCaP-derived positive clones contained the inserts derived from 2 different genes, and the 11 PC3-derived positive clones contained the inserts representing 6 different genes. Some clones were derived from the same gene, as indicated by the same restriction digestion patterns. Other clones showed different restriction patterns, but were found by sequencing analysis to contain the inserts representing the same gene. The different restriction patterns were due to the difference in their insert length.

The results in Table 2 show the reactivity of 10 individual patient sera from serum pool #1. For most clones, there was one patient serum that contains the antibody to the corresponding positive clone. For other clones, two patient sera were found to contain the antibody reactive to

these clones. These results were in consistence with the restriction analysis of the positive clones. The same patient serum reacted to the clones derived from the same genes. Interestingly, the serum from one patient (patient #7) contained the antibodies to 3 distinct cDNA clones. No positive reaction was observed when a pool of 10 sera from normal individuals was used to react with each positive clones, and when a negative clone was used to react with each individual patient serum. This confirmed the specificity of the positive reactions observed between the positive clones and the patient sera.

All positive clones were subcloned to homogeneity, and the nucleotide sequences of the cDNA inserts were determined. The similarity of the identified clones to the known genes in the GenBank database was searched using the BLAST similarity search program. The result is shown in Table 3. Of the 8 genes whose protein products were recognized by prostate cancer patient serum, 5 represented known genes in the GenBank database. Three clones (4, 5 and 7), were previously uncharacterized genes. Clone P1 showed a high homology to a mouse gene, ADP-ribosylation-like factor 6, but no sequence of human homologue was found in the database. Clone LNC1 is homologous to a recently identified human gene KIAA0336 in the GenBank. However, a region of 85 bp was found to be present in the LNC1 cDNA but not in the KIAA0336 cDNA. No functional information is available for KIAA0336 gene.

Clone P9 was designated, prostate antigen recognized and identified by SEREX (PARIS –1). Subsequently, the nucleotide sequence of the PARIS-1 cDNA was determined. Figure 3 shows the complete nucleotide and predicted amino acid sequences of PARIS-1. The PARIS-1 cDNA is 3,256 bp in length and contains a complete open reading frame of 2,751 bp extending from nucleotide position 93 to 2843 encoding for a primary translation product of 917 amino acids with the estimated relative molecular mass of 104 kDa. The sequence flanking the ATG at

position 93 (ATG^{93}) resembles the consensus sequence for functional initiation codons defined by Kozak (Kozak, 1981; 1987). Thus, the ATG^{93} is likely the initiation codon for translation *in vivo*. The calculated pI of the predicted PARIS-1 protein is 8.7, and the predicted molecular weight of the translated protein is approximately 104 kDa. In vitro translation of the PARIS-1 protein showed two distinct bands around 118 kDa (Figure 4). Analysis of the protein sequence for possible post-translational modifications revealed 5 potential N-linked glycosylation sites of the canonical form Asn-X-(Thr/Ser) and a large number of potential phosphorylation sites including 2 cAMP/cGMP-dependent protein kinase phosphorylation sites, 10 protein kinase C phosphorylation sites, 11 Casein kinase II phosphorylation sites and 1 tyrosine kinase phosphorylation sites (Table 4).

To determine the steady-state levels of PARIS-1 mRNA expression, Northern blot analysis was performed on total cellular RNA samples from various human normal and cancer cell lines, including 4 prostate cancer cell lines LNCaP, PC3, DU145, and TSU-Pr1, and a normal prostate epithelial cell line, PreC. A variety of other human normal and cancer cell lines were also included in the assay as shown in Figure 5. Using cloned PARIS-1 cDNA as a probe, we detected a single species of approximate 3.3 kb mRNA in all cells with varied expression levels. Among the cell lines investigated, prostate cancer cell PC3, normal kidney epithelial cell HRE, and glioblastoma cell CRL1620 showed high levels of PARIS-1 mRNA expression. Other prostate cancer cell lines LNCaP, DU145, and TSU-Pr1 showed expression of PARIS-1 mRNA at levels either similar to or lower than that of the normal prostate cell line PreC. These results indicate that the PARIS-1 gene is differentially expressed in prostate cancer cells.

Further SEREX analysis was subsequently performed on both LNCaP, and TSU-Pr1 established cell lines. As before, ZAP Express Phase DNA libraries were constructed and

screened using a pool of sera from prostate cancer patients. One normal was initially used as a control. This effort lead to the identification of several other genes that initially demonstrated reactivity with patient sera (Table 5).

Once clone from LNCaP was reactive against sera, which was then purified and identified as ROK – a kinase implicated in oncogenic signaling required for cell motility and metastatic tumor cell migration. Two clones from TSU-Pr1 and one clone from LNCaP were reactive against another pool of sera, which was subsequently identified as adenylosuccinate lyase (ADSL), a critical enzyme in ATP biosynthesis. This was the first demonstration SEREX-based of ROK and ADSL from prostate tumor cells. These findings suggested that antibodies to ADSL may be associated with cancer, since it was not detectable using several normal sera. Interestingly, ADSL enzymatic activity was reported to increase in certain malignancies. However, the aim of establishing whether or not ROK or ADSL could serve as targets of antibody-based immunotherapy was not accomplished since the grant period ended and further funds were not available to pursue this work.

Several Northern blots of 10-13 cell lines + tissue samples for the analysis of some of the SEREX genes. We concentrated on expression analysis of the two novel genes, GS-3 and GS-7, was completed. Preliminary data on RNA expression of these two genes are shown in Figure 6. Initially, such results suggested that GS-3, and GS-7 in particular, have very high expression in primary tumor tissues (250T, 247T, and 248T, and also in "normal" 250N). These genes appeared not to be restricted to prostate cancer, but expressed at higher levels. However, when we performed an extensive analysis of the frequency of expression (in a total of 43 normal and 64 cancer patient sera) no specificity was noted (Table 6). Some other results were:

GS6. One blot probed with GS6 indicated that some cancer lines (LNCaP and PC3) expressed higher amounts of GS6, but some normal lines (HRE and WI-38) gave the same result.

GS7. GS7 was one of the unknown genes initially discovered by Dr. Shankar via SEREX. However, no proteins reactive with patient sera were identified when lysates from bacteria transformed with a plasmid encoding GS7 was tested by Western blot. Furthermore, an in vitro translation reaction done with this plasmid did not yield a product.

GS8. Most tissues seem to have a fair amount of GS8 expression. PC3 (prostate cancer) overexpresses this gene, while a few other cancer cell lines (U937, 5637 and A378) underexpress GS8.

GS9. Continuing problems with making probes precluded making progress before the grant period expired.

In summary, only two potentially novel genes were identified (GS3 and GS7) After months of anlaysis, we concluded that GS7 was not reactive (or no longer reactive) with patient sera and antibodies to the GS3 gene product were more often found in normal individuals than in prostate cancer patients. The study time ended before we could attempt further analysis of the other, previously identified genes.

<u>Task 3</u>. Determine the range of tissue expression for the protein from which the peptide of interest is derived.

a. Use Northern blotting to assess protein expression in normal prostate tissue as well as other prostate carcinomas.
b. Use Northern blotting to assess protein expression in other tumor types.

Due to the size of prostate tissue obtained from surgical procedures (as well as the small size of the organ itself), it proved impossible to sufficiently large amounts of normal or tumor tissue for analysis. To address this problem, we tried to develop methods for the processing, cultivation, and characterization of the prostate tissue obtained from our affiliate, Northwest Hospital, as well as other sources (Figure 7). Although we utilized all the patient-derived materials possible it was extremely difficult to obtain matched normal and tumor samples from the same donor; we

ended up with but a few such specimens (Table 1a and b). More samples that were anticipated through a productive collaboration with the surgeons and pathologists of Northwest Hospital, and other collaborative institutions, did not materialize.

Due to the aforementioned numerous problems with the T cell-based expression system, we switched to a SEREX approach to antigen discovery. In the time remaining on the grant, we began characterization and tissue expression/distribution studies of the genes that appeared to be novel. The method of analysis we used is summarized in Figure 8. Although we were primarily interested in the potential immunogenicity of the expressed gene products, we did not have sufficient time to complete efforts aimed at producing purified protein using the E. coli expression system (Figure 8). We did make some initial progress (Figure 9). The genes and expression vectors of interest were cut with appropriate restriction enzymes, followed by standard gel electrophoretic analysis. However, we were not able to produce expressed protein that was intended for use in stimulating T cells in vitro, in an effort to generate antigen-specific effectors.

KEY RESEARCH ACCOMPLISHMENTS

- Stable gene modification of an established prostate cell line, LNCaP, with HLA-A1 and –A2.
- Transient gene modification of the established prostate cell lines, DU145 and PC3 – with HLA-A2 – sufficient for MLTC and tumor line distribution analysis of specific T cells.
- Optimization of MLTC
- Generation of cDNA libraries from LNCaP, DU145, and PC3.
- Establishment of a human reagent bank, consisting of patient-derived tissue, cells, and sera.

- Use of T cell expression cloning to identify two potential cloids that may be specifically reactive to a certain region of LNCaP cDNA.
- Establishment of the SEREX technology as a useful means of antigen discovery. This technology did not previously exist at our institution and had to be started from scratch. Within a year, it proved successful.
- Use of SEREX to identify three novel genes whose characterization and potential immunogenicity were assessed.

REPORTABLE OUTCOMES

1. Submission of a patent application on PARIS-1
2. Submission of a manuscript on PARIS-1
3. This award supported the post-doctoral work of Dr. Gopi Shankar.
4. This award supported the post-doctoral work of Dr. Julie McEarchern
5. Funds from this award helped establish a human reagent bank, consisting of patient-derived tissue, cells, and sera.

CONCLUSIONS

It is possible to gene-modify tumor cells to stably or transiently express HLA. This is important when using tumor lines in any immunological studies, since the down-regulation or loss of HLA genes in such cells is a frequent occurrence. While gene-modification of tumor cells in and of itself is well established, we are extending its utility to the discovery of novel

antigens. We have worked out the conditions for MLTC that should provide a greater number of potentially specific T cell cloids/clones from a given experimental set-up. This is not trivial, given the labor- and reagent-intensive nature of MLTC. Our initial efforts with T cell expression cloning produced only two reactive cloids that, upon subsequent screening, may prove to be false positives. However, we have demonstrated the proof of principle for this approach. Due to our efforts to grow prostate-derived TIL, we have a reagent that may be more reactive to expression cloning since such lymphocytes would have had previous exposure to tumor-specific and/or associated antigens.

We anticipated the difficulties experienced in obtaining prostate-derived materials for these studies. Generating autologous tumor lines from patients whose blood samples (and, therefore, immune cells) we collected is a daunting task. As a result, when using the T cell approach to antigen discovery, one is almost exclusively limited to an allogeneic technique using established cell lines. In order to address this disadvantage, our current and future work was amended to include an antibody-based means of antigen discovery: the SEREX approach. An amount of tumor/tissue material insufficient for T cell expression cloning is often adequate for SEREX analysis. Using SEREX, we identified three novel genes not currently in gene databases. Extensive characterization of the GS genes, including one of the two novel genes (GS-3) demonstrated that expression was neither tumor- nor tissue-specific. We did not have time to trouble-shoot problems arising with GS-7.

If time had permitted, we would have continued the analysis of some of the previously identified genes. Some such genes had not been previously associated with prostate cancer. Some such genes had not been shown as immunogenic. It is hoped that those who have attended our presentations at national meetings or will read our manuscripts might follow up on our work.

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Appendix A. Tables

Table 1a. Patient-derived epithelial cell lines being cultured.

Patient No.	Type of Tissue	Growth	Comments
1	prostate	1xT25	
2	prostate	2xT25	
3	prostate	4xT25	fibroblasts potentially taking over
4	normal prostate	3xT25 (explants) 2xT25 (growing cells)	epithelial-like cells growing well
	tumor	3xT25 (explants) 6xT25 (growing cells)	epithelial-like cells growing well
5	normal prostate tumor	2xT25 (explants) 2xT25 (explants)	
6	prostate	2xT25 (explants) 2xT25 (expanded cells)	
7	prostate	3xT25 (explants) 2xT25 (expanded cells)	
8	prostate	2xT25 (explants) 4xT75 (expanded cells)	epithelial/stromal mix epithelial cells
9	prostate	4xT25 (explants) 4xT75 (expanded cells)	epithelial/stromal mix epithelial cells
10	prostate	3xT25 (explants) 4xT75 (expanded cells)	epithelial/stromal mix epithelial cells

11	prostate	4xT25 (explants)	epithelial/stromal mix
12	prostate	4xT25 (explants) 2xT75 (expanded cells)	epithelial/stromal mix epithelial cells
13	prostate	3xT25 (explants)	epithelial/stromal mix

Table 1b. Patient-derived TIL in culture.

Patient No.	Type of Tissue	Aliquots Frozen ($>1x10^6$/vial)
1	prostatectomy	none
2	prostatectomy	none
3	prostate	2
4	prostate	1
5	prostate	5
6	prostate	none
7	prostate	2
8	prostate	none as yet (slow growth)
9	prostate	none as yet (slow growth)
10	prostate	none as yet (slow growth)
11	prostate	none as yet (slow growth)
12	prostate	none as yet (still at explant stage)
13	prostate	none as yet (still at explant stage)
14	prostate	none as yet (still at explant stage)

Table 2. Reaction of positive clones to individual patient serum

	Prostate Cancer Patient Serum (Stage D2)									
Clone #	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	Normal
P1							+				
P2						+					
P3						+					
P4						+					
P5						+					
P6					+						
P7		+							+		
P8						+					
P9		+							+		
P10						+					
P11		+							+		
P(-)											

Table 3. Identities of the SEREX-identified clones from prostate cancer cell lines.

Clone ID	Source	Homologous Genes in GenBank	Comments
P1	PC3	Mouse ADP-ribosylation-like factor 1 (ARL1)	No human homologue
P2	PC3	Dihydrolipoamide dehydrogenase, (LPD, DLD)	
P5	PC3	Translation initiation factor 3 (47 kD subunit), (eIF3-p47)	
P8	PC3	None	New gene
P9	PC3	None	New gene
P16	PC3	Fanconi anemia complementation group A, protein (FAA)	
LNC1	LNCaP	A new gene with unknown function	Unknown function
LND4	LNCaP	Heterogeneous nuclear M4 protein	

Table 4. Posttranslational modification sites on hPCA-1 protein

Posttranslational modification sites	Amino acid positions	Conserved amino acids
N-linked glycosylation Sites	205-207	NIS
	288-290	NNT
	301-303	NRT
	675-677	NRT
	706-708	NPT
cAMP- and cGMP-dependent protein kinase phosphorylation sites	343-346	KRAS
	896-899	RRAS
Protein kinase C phosphorylation sites	21-23	SAR
	121-123	TLK
	207-209	SLK
	267-269	SPK
	324-326	SQK
	391-393	SLR
	422-424	SEK
	768-770	SEK
	848-850	SRK
	899-901	SRR
Casein kinase II phosphorylation sites	21-24	SARD
	83-86	TAQD
	231-234	TGHE
	303-306	TAQE
	324-327	SQKE

	391-394	SLRE
	558-561	SKYD
	567-570	TVPD
	690-693	SFPD
	756-759	SQVD
	910-913	SEDE
Tyrosine kinase phosphorylation sites	808-815	RVWDAFLY

Table 5. Identities of the SEREX-identified Clones from Prostate Cancer Cell Lines

Clone ID	Source	Homologous Genes in GenBank	Comments
GS-1/2/5	TSU-Pr1	Adenylosuccinate (ADSL)	New in prostate cancer
GS-3	LNCaP	None	New gene
GS-4	LNCaP	Rho-associated coiled-coil containing protein kinase (ROK1)	New in prostate cancer
GS-6	LNCaP	A new gene with unknown function (EST)	New in prostate cancer
GS-7	LNCaP	None	New gene
GS-8	LNCaP	Golgin (human trans-golgi peripheral membrane protein)	New in prostate cancer

Table 6. SEREX Summary of GS genes

Gene	Frequency Cancer vs. Normal (%)	
GS-1	**3.1**	**0.0**
GS-3	15.6	32.6
GS-4	21.9	14.0
GS-6	18.8	11.6
GS-7	not reactive – protein not translated	
GS-8	10.9	16.3
GS-9	6.3	14.0

A total of 64 cancer patient sera and 43 normal donor sera were screened for each gene.

Appendix B. Figures

Figure 1. Processing/Cultivation/Characterization of prostate tissue intended for T cell or SEREX antigen identification methods.

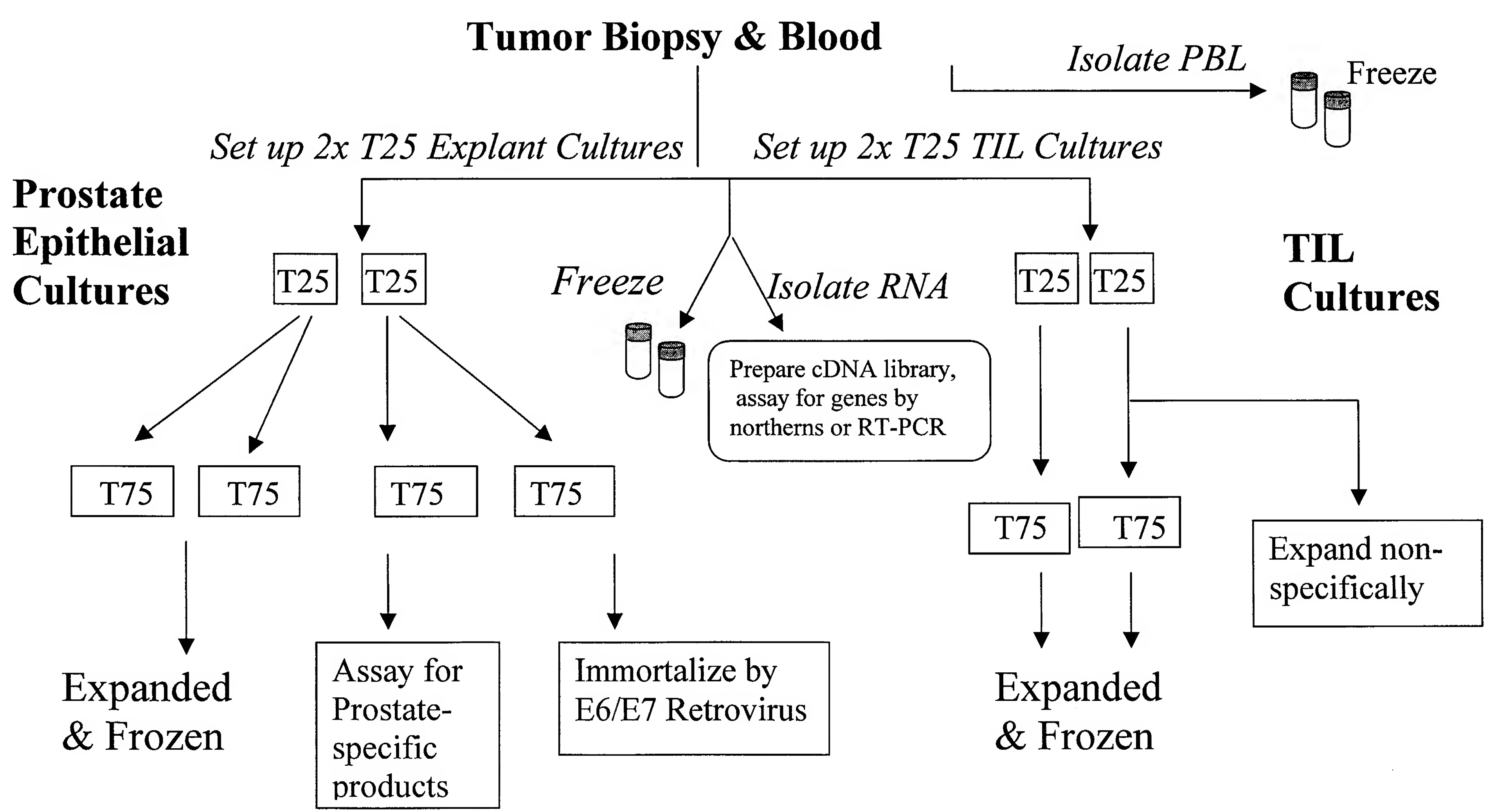

Figure 2. Overview of our approach to SEREX (Serological Identification of Antigens by Recombinant Expression Cloning)

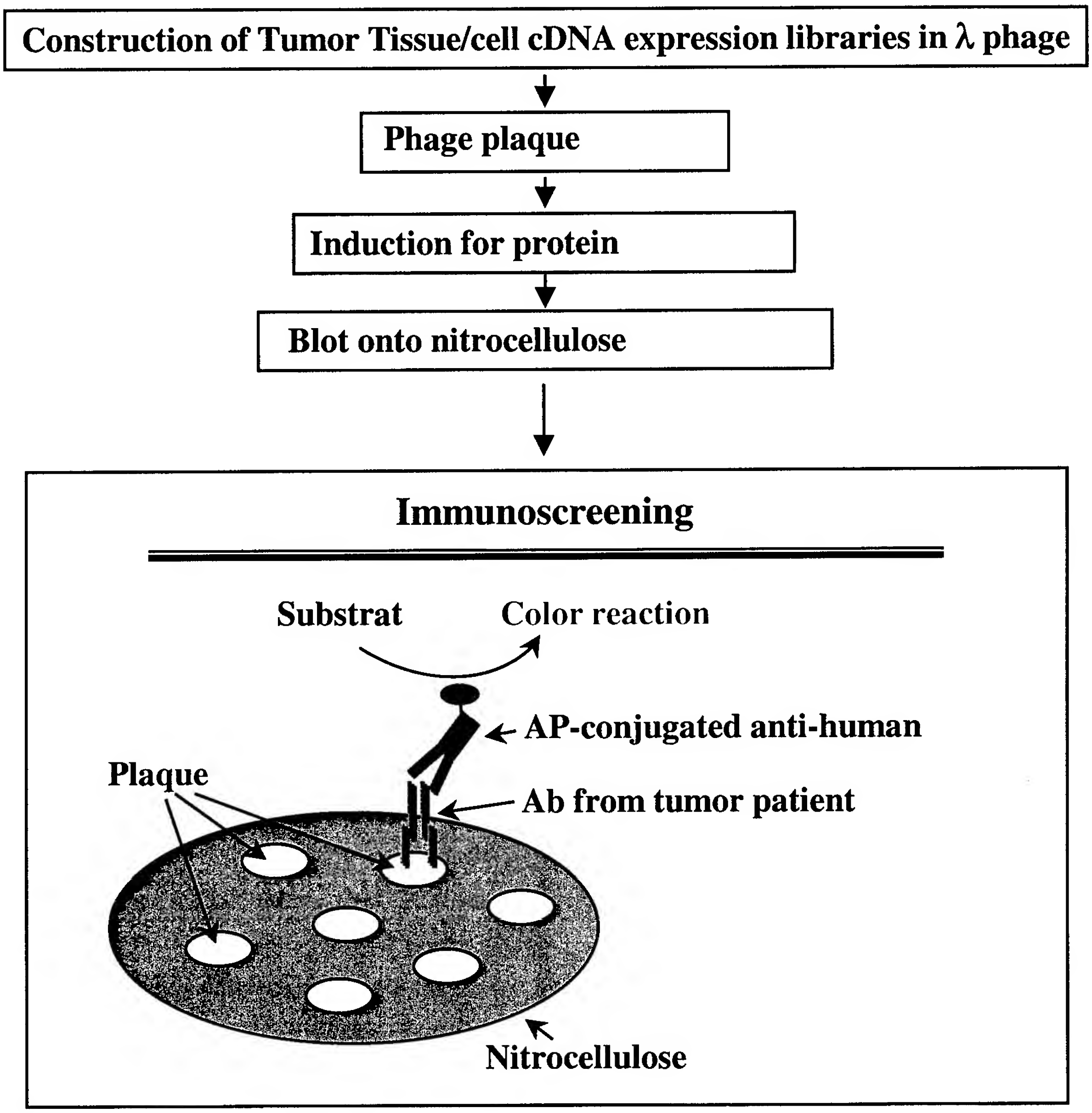

Figure 3. Nucleotide and predicted amino acid sequences of PARIS-1. Human PARIS-1 was identified from the human prostate cancer cell line, PC3. The nucleotide sequence of PARIS-1 cDNA is 3,256 bp in length containing a complete open reading frame of 2,751 bp encoding for a protein of 917 amino acids. The five underlined amino acids indicate potential N-linked glycosylation sites on the protein.

```
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agtccagctcctctgcccctgggtccgaagagtctgccagggatccacaggtgccgcctc  180
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```

Fig. 4. In vitro translation of the PARIS-1 protein. Proteins were synthesized from the plasmids containing PARIS-1 cDNA, P8 cDNA, or no cDNA in a transcription/translation coupled reticulocyte lysate system as described in Materials and Methods. The translation products were separated in a 10% SDS-PAGE, vacuum dried, and exposed X-ray film. The sizes of the molecular markers were indicated.

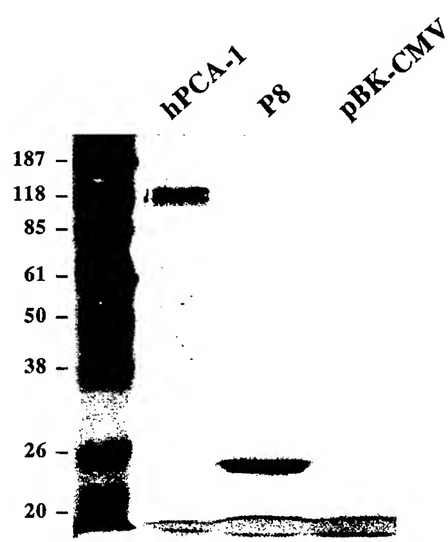


Figure 5. Northern blot analysis of PARIS-1 mRNA expression in human normal and cancer cell lines. Total cellular RNA was isolated from human normal and cancer cell lines as indicated. LNCaP, PC3, DU145, and TSU-Pr1 are human prostate cancer cell lines. PreC is a normal human prostate epithelial cell line. Other cell lines include HRE (normal renal epithelial), WI-38 (normal lung fibroblast), A1N4 and MCF10A (normal breast epithelial), ZR-75-1 (breast cancer), HT1080 (fibrosarcoma), U937 (leukemia), 5637 (bladder carcinoma), CRL1620 (glioblastoma), and A378mel (melanoma). The RNA was hybridized sequentially with cDNA probes for PARIS-1 and the control gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and visualized by autoradiography (A). The PARIS-1 mRNA levels were quantified by densitometric scanning of the hybridized bands and normalized to GAPDH mRNA levels (B). The size of the hybridized PARIS-1 mRNA is indicated.

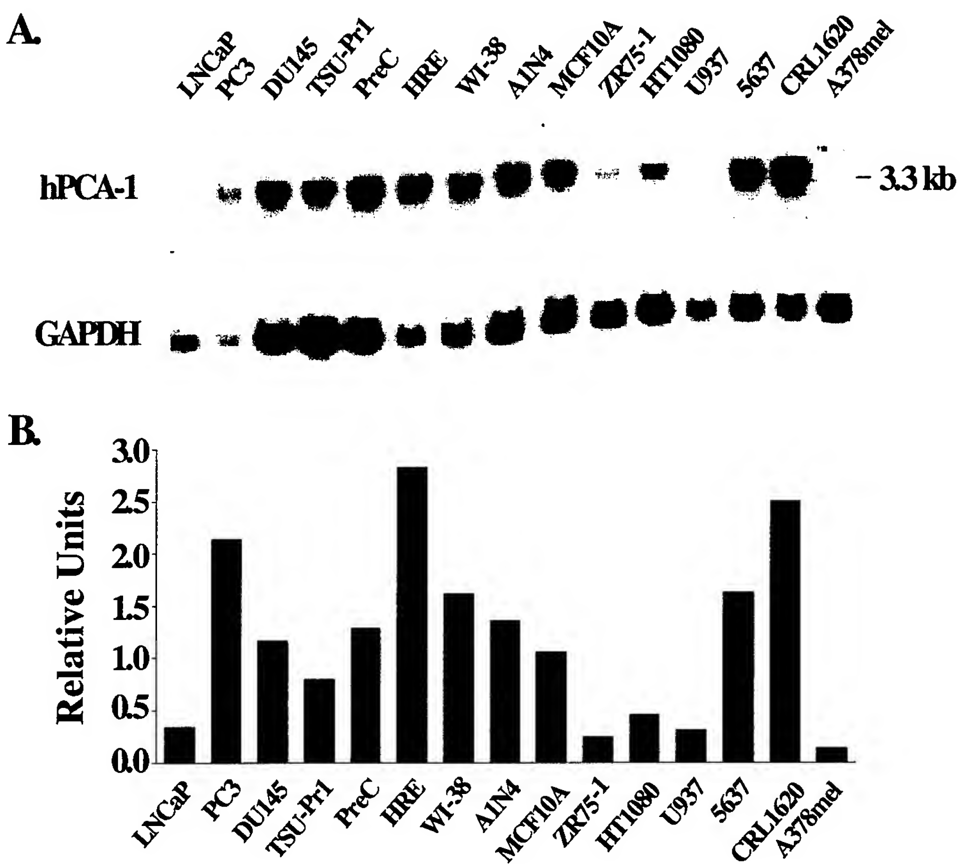
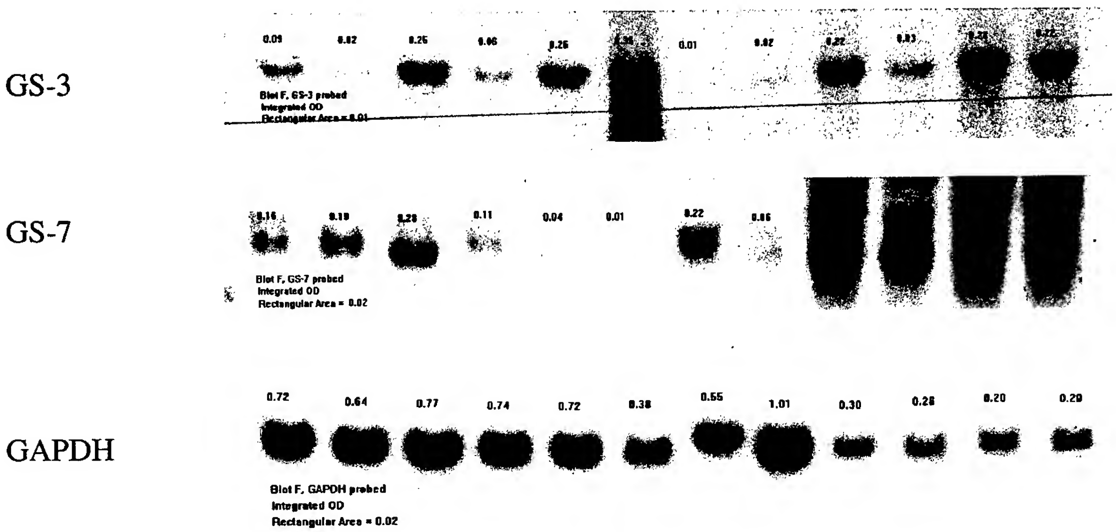


Figure 6. Expression analysis of the two novel genes, GS-3 and GS-7



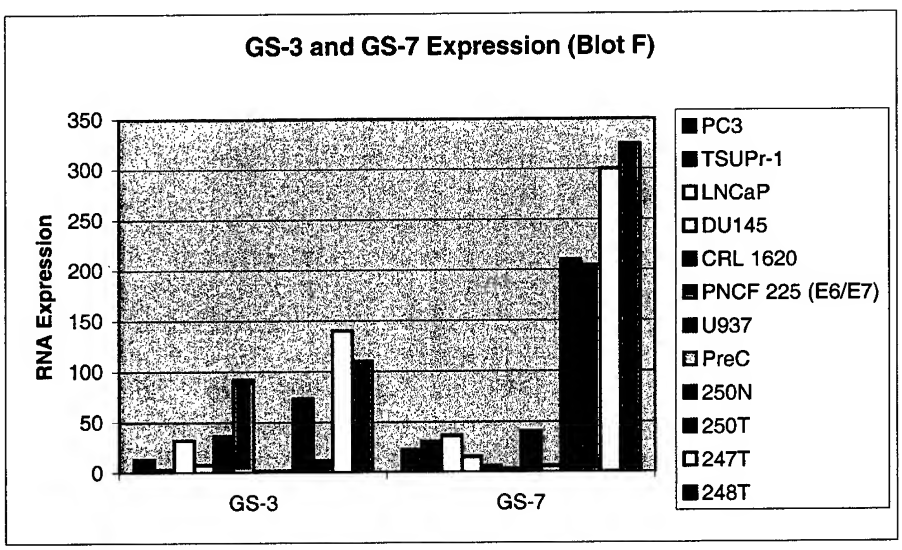


Figure 7. Characterization approach for genes and antigens identified by T cell expression cloning as well as SEREX.

RNA expression

- **Northern blot** — **Cell lines**; **RNA blots**
- **RT-PCR + Southern blot** — **Tissue RNA (NWB tissue bank, NWH tissue center)**; **Matched cDNA pairs**
- **In situ Hybridization** — **Tissue slides**

Protein expression

- **Antibody/peptide library** — **Tissue slides**

Figure 8. Approach for the expression of proteins in E. coli identified by T cell expression cloning or SEREX

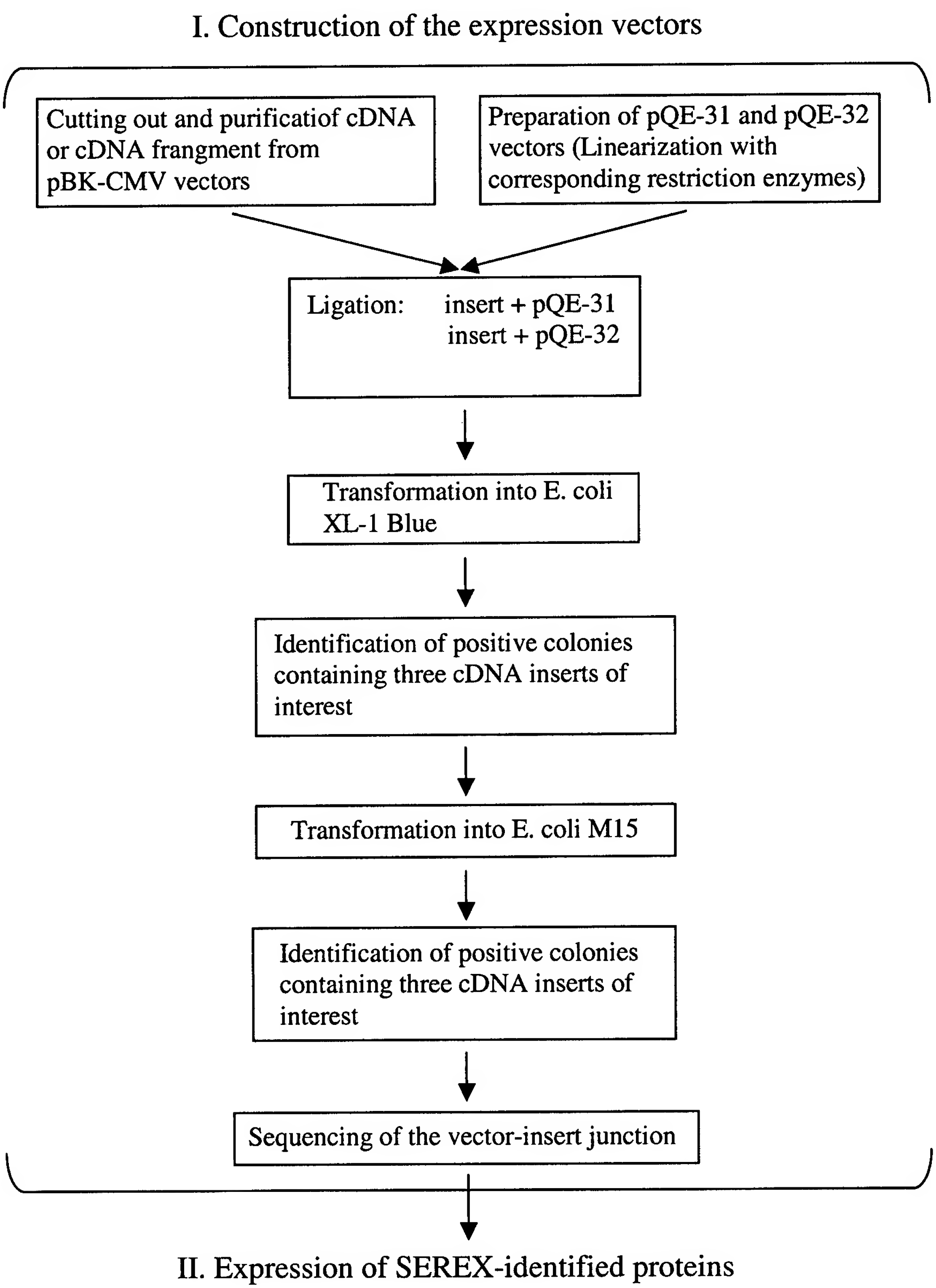
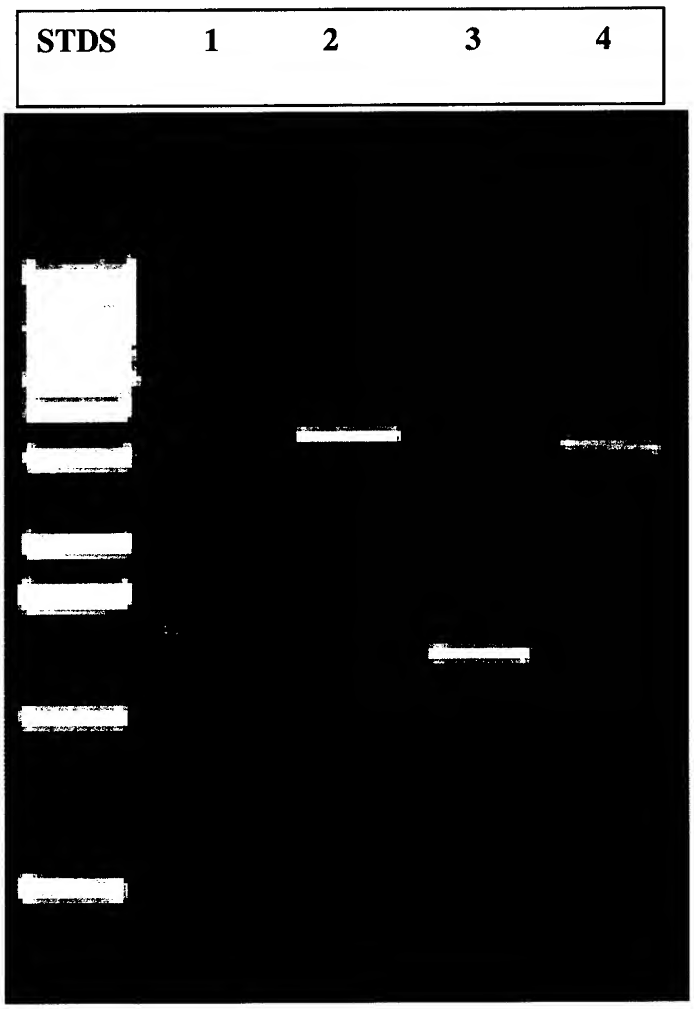


Figure 9. Preparation of P1, P2 inserts and pQE31, 32 vectors for the generation of expression vectors.



Lanes:

1. P1 insert (BamH1/Kpn1)

2. pQE32 vector (BamH1/Kpn1)

3. P2 insert (BamH1/Kpn1)

4. pQE31 vector (BamH1/Kpn1)